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Genetic Basis for Morphological Variation in the Zebrafish *Danio rerio*: Insights from a Low-Heterozygosity Line

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Abstract: Data variability complicates reproducibility and the interpretation of experimental results. Different animal models have been employed to decrease variability to enhance experimental power. However, variation frequently persists among and within strains/lines. In zebrafish (*Danio rerio*), inbred lines (e.g., NHGRI-1) derived from wild-type lines have been produced to greatly decrease genetic variation, with the goal of providing better understanding of genetic backgrounds that may influence the experimental outcome of studies employing such lines. We hypothesized that variations in morphological phenotypes shaped by environmental stressors early in development are correlated with the intrinsic degree of genetic variability of zebrafish lines. We compared morphological variability (yolk–chorion ratio, body mass, embryo mass, total length, condition factor, and specific growth rate) in wild-type AB and NHGRI-1 zebrafish lines as a function of their responses to altered temperature and oxygen availability during the first 7 days post-fertilization. Overall, both lines showed similar developmental trajectories for yolk–chorion ratio, embryo mass, and total length. Additionally, condition factor and specific growth rate showed similar responses within each line, regardless of temperature and hypoxia. Importantly, the coefficient of variation for each variable was significantly lower in NHGRI-1 than AB larvae for 151 of 187 assessed morphological endpoints. Thus, the low-heterozygosity NHGRI-1 zebrafish line can be useful for decreasing inter-individual variation in morphological responses to environmental stressors, thereby aiding in the interpretation of results and enhancing experimental reproducibility.

Keywords: variation; low heterozygosity; condition factor; specific growth rate; coefficient of variation; NHRGI-1 zebrafish

Key Contribution: Using animal models with low genetic variability during experimentation facilitates reproducibility. Here, we show how the morphology of early NHGRI-1 zebrafish (low-heterozygosity line) has less variation than wild-type AB zebrafish.

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1. Introduction

The reproducibility of experiments is of increasing concern in scientific research [1]. In a 2016 survey, ~70% of researchers stated that they were not able to reproduce data from other researchers and, occasionally, their own [2]. The reliability of data acquisition and processing depends on numerous factors, including researcher biases, experimental power, number of studies about a certain topic, insufficient reporting of methods, and animal husbandry—that is, variation produced by how data are collected by researchers [3–5]. However, a lack of reproducibility can also result from differences in phenotypes or genotypes between individuals in the experiments [4,6–9].

The zebrafish (*Danio* rerio) is a widely used animal model for biological and medical research (among other fields) because of their similarities with human genomes, transparent embryos to assess development, low husbandry costs, etc.—for a recent review, see [10–13]. However, the lack of uniformity in zebrafish genetic backgrounds has limited

their definition as 'inbred strains' (to reduce genetic variability) or 'outbred stocks' (to increase genetic variability), both of which limit their reproducibility [7]. Zebrafish from natural populations show higher genetic variation than laboratory lines [14], and the genetic background of zebrafish lines varies among laboratories and/or facilities [15]. Differences in the swimming performance, behavior, growth, and regulation of gene expression have been identified among different zebrafish lines [16–23].

The use of homozygous strains/lines is generally employed in experimentation which requires low variability in their genetic background to facilitate the reproducibility of results because of their lower heterozygosity and long-term stability [24,25]. Despite the existing evaluation of different traits between homozygous and heterozygous strains/lines (see references above), most information is related to genetic studies including single-nucleotide polymorphism (SNP) variation, assessing copy number variants between zebrafish lines, the role of xenosensors in nuclear receptor function, building genetic repositories, and comparing genetic backgrounds from wild and inbred populations, [14,15,25–28], with little study in this context of morphology, physiology, or behavior.

The AB line was a zebrafish line produced for laboratory work, and it is still widely used [7]. AB fish are believed to come from the ornamental fish trade in North America, farmed in Southeast Asia and the southeast USA [29]. Their genetic variability is high because of the great number of sublines globally available [7]. The need for a strain with a better-understood genetic background has led to the generation of the NHGRI-1 line, which possesses a lower degree of heterozygosity than wild-type lines (~10–15% from AB wild-type) [7,30].

The aim of the current study was to assess the variability in yolk–chorion ratio, body mass, embryo mass, total length, condition factor, and specific growth rate in AB and NHGRI-1 zebrafish lines as a function of temperature and oxygen availability during the first 7 dpf (day post-fertilization). We hypothesized that key characteristics of morphological phenotypes would show lower variation in embryos and larvae of the inbred NHGRI-1 line compared to the wild-type AB zebrafish. We tested this hypothesis specifically in the early developmental stages to eliminate potential epigenetic effects or uncontrolled environmentally induced variation accumulated over the period of growth throughout the first seven days of development, and because zebrafish are an important model system of ontogenetic development [31,32].

2. Materials and Methods

2.1. Fish Sources and Breeding

Embryos and larvae of AB and NHGRI-1 zebrafish were obtained from colonies of breeding adults held at the Department of Biological Sciences, University of North Texas. AB fish were originally purchased from Ekkwill Waterlife Resources (Ruskin, FL, USA), while adult NHGRI-1 fish were purchased from the Zebrafish International Resource Center (ZIRC) (Eugene, OR, USA). One hour after breeding, fertilized eggs were divided into experimental groups (described below). Larvae were fasted and maintained in an adequate medium for rearing embryonic and larval fish—E3 medium [31,33]—throughout experimentation. Fifty percent of the medium was gently replaced daily. Dead embryos/larvae were removed using a plastic pipette. Larvae at all stages were maintained in 14/10 h light and darkness, respectively.

2.2. Experimental Design

Five groups were generated for each of the two lines: (1) control at 28 °C in normoxia at ~7.8 mg L^{-1} O_2 (~21 $kPaO_2$), (2) low temperature (23 °C in normoxia), (3) high temperature (33 °C in normoxia), (4) moderate hypoxia at ~4.9 mg L^{-1} O_2 (~13 $kPaO_2$at 28 °C), and (5) severe hypoxia at ~3.7 mg L^{-1} O_2 (~10 $kPaO_2$ at 28 °C). Each group contained three replicates and 100 viable embryos were utilized per replicate. Individuals were exposed to each condition from 1 hpf (hour post fertilization) through 7 dpf. Control and high temperature groups were maintained in incubators (Hera Cell 240i, Fisher Scientific,

Pittsburgh, PA, USA) provided with airflow throughout experimentation. Hypoxic groups were maintained in incubators (MCO-5M-PA, Panasonic, American Laboratory Trading, East Lyme, CT, USA) supplied with a controlled mix of air and nitrogen generating the required oxygen level. Air and nitrogen were regulated with flow meters (Lab-Crest Century 100H, Cole-Palmer, Vernon Hills, IL, USA) and empirically adjusted to ~4.86 and 3.73 mg L^{-1} O_2 for moderate and severe hypoxia, respectively. Oxygen levels were assessed every 24 h using a Beckman oxygen analyzer (OM-11, Beckman Coulter Inc., Brea, CA, USA) calibrated to 100% air saturation.

All procedures in this study were carried out under approval of the Institutional Animal Care and Use Committee of the University of North Texas (IACUC—21002).

2.3. Assessed Variables

All morphological variables were measured every 24 h throughout experimentation. Fifteen fish were sampled to assess each variable. Embryos were mechanically dechorionated under a dissecting microscope using fine-tip forceps before processing, except for Yolk/Chorion ratio calculations. Embryos and larvae were fixed in 10% aqueous buffered zinc formalin (Z-Fix; Electron Microscopy Sciences, Hatfield, PA, USA) for 24 h, rinsed in 1X PBS, and immediately processed.

Yolk/chorion Ratio (YC_R). Embryos were photographed under a dissecting microscope before fixation with a camera (Leica MDC6200) using the software Leica Application Suite V 3.4.0. Images were analyzed in the software ImageJ 1.53k [34]. The area of the egg and the area occupied by the embryo were calculated. The area of the embryo was divided by the total area of the egg and multiplied by 100 to obtain YC_R as a percentage.

Body Mass (*BM*). Embryos and larvae were weighed using a Mettler Toledo XA105 digital scale to determine 'body mass'. Then, the yolk was removed from the embryos/larvae, which were then re-weighed under the same protocol to obtain the 'embryo mass' (*EM*).

Total length (L_T). Embryos and larvae were photographed and measured using ImageJ 1.53k to obtain L_T in millimeters.

Condition Factor (*K*). *K* was calculated as $K = 100 \times BM \div {L_T}^3$ [35].

Specific growth rate (*SGR*). Percentage growth per day was calculated as $SGR = 100 \times (\ln EM_t - \ln EM_i) \div t$ [36], where EM_t represents wet body mass of the embryo at each specific endpoint, EM_i represents the wet mass of the embryo at 24 hpf, and t represents time.

2.4. Statistics

Data were separated as temperature and hypoxia experiments for analysis. The graphical representation of results is presented only for the integrated variables condition factor (*K)* and specific growth rate (*SGR*); the rest are presented as tables. Changes in variables as a function of developmental time and experimental groups were assessed with two-way ANOVAs. Multiple comparison Holm–Sidak tests [37,38] were carried out to determine differences within time and experimental groups.

Variability between fish lines for each variable at a given time was quantified by calculating the coefficient of variability, C_V, associated with the mean values of each morphological variable. Microsoft Excel 2023 was used to calculate C_V from the equation $C_V = \sigma' \div \overline{x}$; where σ' represents the standard deviation and $\overline{x}$ represents the mean [39]. Differences between C_V of wild-type AB and NHGRI-1 lines were tested for significance using a Z-test. Two-way ANOVAs at a 0.05 confidence level and figures were generated in SigmaPlot V.12.3.

3. Results

3.1. Body and Embryo Mass

Body mass in the AB control embryos at 1 dpf was 248 ± 8 mg, rising to 314 ± 11 mg in 7 dpf larvae. All experimental groups showed gradual increases in body mass from 1 to 7 dpf ($p < 0.001$, Table 1), apart from the high-temperature groups from both lines. Larvae from the high-temperature groups from both lines showed an initial increase

($p < 0.001$) in *BM* (301 ± 8 to 337 ± 13 µg in AB larvae; 243 ± 4 to 300 ± 5 µg in NHGRI-1 larvae). However, by the end of the experiment at 7 dpf, both lines showed significantly decreased ($p < 0.001$) *BM* (293 ± 9 µg, 276 ± 5 µg) compared to their highest values (Table 1). Significant differences were more evident in NHGRI-1 fish than in AB individuals. By the end of the hypoxic experiments, larvae from severe hypoxia treatments showed significantly lower ($p < 0.001$) *BM* compared to controls (266 ± 3 and 286 ± 4 µg, respectively) (Table 1).

Table 1. Morphological variables determined in AB and NHGRI-1 lines of zebrafish (*Danio rerio*). Data per day and experimental group are presented as mean ± SEM ($n = 15$). Asterisks on values in bold represent differences from controls at any given day. *p* values after the day 7 entry indicate differences within each experimental group across developmental time.

TEMP (°C)	O_2 (mg L^{-1}))	AGE (dpf)	Yolk/Chorion Ratio (%)		Body Mass (µg)		Embryo Mass (µg)		Total Length (mm)	
			WT AB	NHGRI-1	WT AB	NHGRI-1	WT AB	NHGRI-1	WT AB	NHGRI-1
28	7.8	1	38 ± 1	43 ± 1	284 ± 8	276 ± 4	120 ± 3	110 ± 2	3.1 ± 0.1	2.5 ± 0.03
		2	55.8 ± 2	51.6 ± 1.6	278 ± 10	266 ± 5	150 ± 4	120 ± 2	3.3 ± 0.1	2.8 ± 0.02
		3	63.7 ± 2.2	-	305 ± 9	256 ± 4	180 ± 7	120 ± 2	3.9 ± 0.1	2.9 ± 0.03
		4	-	-	315 ± 9	268 ± 3	230 ± 7	160 ± 2	4.2 ± 0.2	3.2 ± 0.04
		5	-	-	349 ± 12	285 ± 4.	280 ± 10	190 ± 3	4.2 ± 0.1	3.4 ± 0.03
		6	-	-	329 ± 15	270 ± 6	290 ± 13	220 ± 5	4.2 ± 1	3.5 ± 0.04
		7	-	-	314 ± 11	286 ± 4	300 ± 10	250 ± 4	4.4 ± 0.1	3.9 ± 0.05
			$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
23	7.8	1	**47 ± 1.4** *	39.9 ± 1.0	290 ± 7.2	**215 ± 3** *	**120 ± 7** *	90 ± 2.	3.2 ± 0.10	2.3 ± .0.02
		2	54.1 ± 1.3	54.9 ± 1.7	306 ± 7.3	**215 ± 4** *	**130 ± 4** *	**130 ± 2** *	**3.4 ± 0.1** *	2.4 ± 0.03
		3	55.5 ± 2.2	53.8 ± 1.7	291 ± 8.5	**227 ± 5** *	**130 ± 6** *	**130 ± 3** *	**3.7 ± 0.1** *	2.9 ± 0.03
		4	64.1 ± 2.3	-	297 ± 5.6	**234 ± 5** *	**180 ± 6***	**160 ± 3** *	**3.8 ± 0.1** *	**3.2 ± 0.03** *
		5	-	-	**306 ± 5.9** *	**253 ± 6** *	**210 ± 6** *	**200 ± 5** *	3.8 ± 0.1	3.2 ± 0.04
		6	-	-	339 ± 11.2	269 ± 4	280 ± 11	**230 ± 4** *	4.1 ± 0.1	3.2 ± 0.04
		7	-	-	334 ± 10	279 ± 4.	**300 ± 10** *	**260 ± 4** *	4.1 ± 0.1	3.4 ± 0.05
			$p < 0.001$	$p < 0.001$	$p < 0.006$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
33	7.82	1	**49.8 ± 2.2** *	**53 ± 2** *	301 ± 7.9	**243 ± 3.8** *	120 ± 4	**120 ± 2.1** *	3.43 ± 0.1	2.7 ± 0.03
		2	-	-	295 ± 7.2	**300 ± 4.8** *	170 ± 5	200 ± 3.3	**3.98 ± 0.1** *	**3.1 ± 0.03** *
		3	-	-	317 ± 8.5	**273 ± 5.8** *	**210 ± 6** *	**180 ± 3.7** *	**4.17 ± 0.1** *	**3.3 ± 0.03** *
		4	-	-	**337 ± 11.3** *	**298 ± 3.8** *	260 ± 9	220 ± 3.2	**4.47 ± 0.1** *	**3.4 ± 0.04** *
		5	-	-	327 ± 9.3	**300 ± 5.2** *	280 ± 8	260 ± 4.5	**4.51 ± 0.1** *	**3.5 ± 0.04** *
		6	-	-	337 ± 12.7	284 ± 3.4	**300 ± 11** *	**250 ± 3.3** *	**4.59 ± 0.1** *	**3.7 ± 0.04** *
		7	-	-	**293 ± 8.7** *	276 ± 5.2	280 ± 8	**270 ± 5.6** *	**4.65 ± 0.2** *	**4.2 ± 0.04** *
			$p < 0.001$	-	$p < 0.026$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
28	4.9	1	38.1 ± 1.5	42.3 ± 2.2	294 ± 8.8	**242 ± 5.7** *	**100 ± 7** *	**80 ± 2.2** *	3.08 ± 0.1	2.1 ± 0.02
		2	52 ± 1.6	55.1 ± 2.3	279 ± 7.7	**233 ± 3.0** *	**110 ± 6** *	**100 ± 1.7** *	3.30 ± 0.1	2.8 ± 0.03
		3	**43.9 ± 2.4** *	50.5 ± 1.7	295 ± 7.8	229 ± 4.1	**130 ± 8** *	**100 ± 1.9** *	**3.40 ± 0.2** *	**2.9 ± 0.04** *
		4	-	-	293 ± 7.6	**209 ± 3.0** *	**170 ± 4** *	120 ± 1.9	**3.63 ± 0.2** *	**3 ± 0.04***
		5	-	-	**297 ± 7.5** *	**245 ± 4.5** *	**190 ± 5** *	150 ± 3.0	**3.80 ± 0.1** *	**3.2 ± 0.04** *
		6	-	-	303 ± 7.0	260 ± 5.3	**230 ± 9** *	**190 ± 4.0** *	4.11 ± 0.1	3.4 ± 0.05
		7	-	-	312 ± 9.7	275 ± 3.5	**270 ± 9** *	**230 ± 2.8** *	4.20 ± 0.1	3.6 ± 0.04
			$p < 0.001$	$p < 0.001$	$p = 0.189$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
28	3.7	1	39.3 ± 1.5	46.2 ± 1.2	296 ± 7.0	**219 ± 5** *	**110 ± 4** *	**80 ± 1.7** *	3.0 ± 0.1	2.2 ± 0.02
		2	54.1 ± 2	50.7 ± 1.8	288 ± 6.4	**220 ± 4.1** *	**120 ± 6** *	**90 ± 2.0** *	3.2 ± 0.1	2.5 ± 0.04
		3	**42.4 ± 2** *	50.6 ± 3.4	291 ± 6.5	210 ± 3.4	**140 ± 5** *	**90 ± 1.7** *	**3.3 ± 0.1** *	**2.9 ± 0.04** *
		4	-	-	302 ± 9.4	**209 ± 3.6** *	**170 ± 1** *	120 ± 2.4	**3.5 ± 0.1** *	**3.0 ± 0.04** *
		5	-	-	**293 ± 6.3** *	**250 ± 4.4** *	**180 ± 6** *	150 ± 2.6	**3.6 ± 0.1** *	**3.1 ± 0.05** *
		6	-	-	291 ± 7.2	263 ± 4.6	**210 ± 7** *	**180 ± 3.2** *	3.8 ± 0.1	3.3 ± 0.05
		7	-	-	320 ± 9.6	**266 ± 3.2** *	**260 ± 9** *	**210 ± 1.9** *	**4.0 ± 0.1** *	**3.3 ± 0.04** *
			$p < 0.001$	$p = 0.264$	$p = 0.217$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$

The control larvae and the low- and high-temperature (only NHGRI-1), moderate hypoxia, and severe hypoxia larval groups showed significant ($p < 0.001$), gradual increases in *EM*. In contrast, high-temperature AB embryos and larvae increased (120 ± 4 to 300 ± 11 µg) from 1 to 6 dpf, but by 7 dpf, the *EM* of high-temperature larvae decreased (280 ± 8 µg; $p < 0.001$; Table 1). By the end of the experiments, AB larvae from the high-temperature, moderate hypoxia, and severe hypoxia treatments showed significantly ($p < 0.001$) lower *EM* (280 ± 8 µg; 270 ± 9 µg, and 260 ± 9 µg, respectively) compared to control larvae (300 ± 10 µg; Table 1). Low- and high-temperature NHGRI-1 larvae showed increased *EM* (260 ± 4.2 µg and 270 ± 5.6 µg, respectively) compared to controls (250 ± 4 µg), while larvae from mild and severe hypoxia showed significantly ($p < 0.001$) decreased *EM* (230 ± 38 µg and 210 ± 2 µg, respectively) (Table 1).

3.2. Total Length

The total length in control embryos-to-larvae increased from 2.5 ± 0.03 mm at 1 dpf to 4.4 ± 0.1 mm at 7 dpf. Continuous, significant ($p < 0.001$) increases in L_T occurred in all experimental groups across developmental time. After 7 dpf, the highest L_T was observed in high-temperature larvae from both lines (4.7 ± 0.2 mm in AB larvae, 4.2 ± 0.04 mm in NHGRI-1; $p < 0.001$; Table 1). Neither high-temperature nor moderately hypoxic larvae showed a significant difference ($p > 0.05$) (4.1 ± 0.1 mm in AB, 3.4 ± 0.1 mm in NHGRI-1) from their controls, which were themselves significantly ($p < 0.001$) different (4.4 ± 0.1 mm in AB, 3.9 ± 0.1 in NHGRI-1; Table 1). AB and NHGRI-1 larvae from severe hypoxia showed significantly ($p < 0.001$) lower L_T (4.0 ± 0.1 mm and 3.3 ± 0.04 mm, respectively) than their respective controls by 7 dpf (Table 1).

3.3. Yolk/Chorion Ratio

The yolk/chorion ratio in control AB embryos at 1 dpf was 38 ± 1%, rising to 56 ± 2% at 2 dpf and peaking at 64 ± 2 before hatching at 3 dpf (Table 1). Both low- and high-temperature AB embryos had increased YC_R (47 ± 1.4 and 50 ± 2%, respectively) compared to their controls by 1 dpf. Prior to hatch, no differences ($p > 0.05$) were observed in YC_R between low-temperature AB embryos and their controls. Moderate and severe hypoxic AB embryos showed significantly lower YC_R than their controls from 1 to 3 dpf, ranging from 38 ± 1.5 to 54 ± 2%. Control NHGRI-1 embryos had a YC_R of 43 ± 0.7% by 1 dpf and 52 ± 1.6 prior to hatching (Table 1). By 1 dpf, only high-temperature NHGRI-1 embryos showed significant differences compared to their controls (53 ± 2 vs. 43 ± 0.7%). No significant differences ($p > 0.05$) in YC_R were observed among NHGRI-1 groups prior to hatch. No significant differences ($p > 0.05$) across developmental time were observed in any NHGRI-1 group, except for the high-temperature group.

Additionally, the time to hatch in control AB zebrafish was ~4 dpf, and it was delayed by low temperature and accelerated by high temperature (~5 and ~2 dpf, respectively). Hypoxia did not affect the time to hatch in AB zebrafish (Table 1). Similarly, control NHGRI-1 zebrafish hatched at ~3 dpf. Time to hatch was delayed (~4 dpf) in the low temperature and both hypoxic groups, while high temperature decreased the time to hatch to ~2 dpf (Table 1).

3.4. Condition Factor

The condition factor in control larvae decreased from ~1.0 at 1 dpf to ~0.5 at 7 dpf in AB individuals and from ~1.9 (1 dpf) to ~0.5 (7 dpf) in NHGRI-1 embryos and larvae. Gradual decreases in *K* occurred during development in larvae from all experimental groups (Figure 1A–D). In general, no significant differences ($p > 0.05$) occurred among experimental groups after low/high temperature or hypoxia exposure in AB larvae (Figure 1A,B). At 1 dpf, NHGRI-1 embryos from low- and high-temperature groups showed significantly ($p < 0.001$) lower *K* (1.7 ± 0.1 and 1.2 ± 0.04, respectively) compared to control embryos (1.9 ± 0.1; Figure 1C). At the end of the exposures on 7 dpf, low-temperature NHGRI-1 larvae showed the highest *K* (0.7 ± 0.03), and high-temperature NHGRI-1 larvae showed the lowest K (0.4 ± 0.01; $p < 0.001$; Figure 1C). Among the hypoxic exposures in NHGRI-1 embryos and larvae, significant differences ($p < 0.001$) were observed only at 1, 2, and 7 dpf (Figure 1D). By 1 dpf, control embryos exhibited significantly ($p < 0.001$) lower *K* (1.9 ± 0.1) than embryos from moderate and severe hypoxia (2.5 ± 0.2 and 2.2 ± 0.1, respectively; Figure 1D). The highest *K* at 2 dpf was observed in severe hypoxia embryos (1.4 ± 0.1; $p < 0.001$), while moderate hypoxia embryos and controls were not significantly ($p > 0.05$) different (1.1 ± 0.04 and 1.2 ± 0.04, respectively; Figure 1D). By 7 dpf, only the severely hypoxic larvae had significantly ($p < 0.001$) different *K* from controls (0.7 ± 0.02 and 0.5 ± 0.02, respectively; Figure 1D).

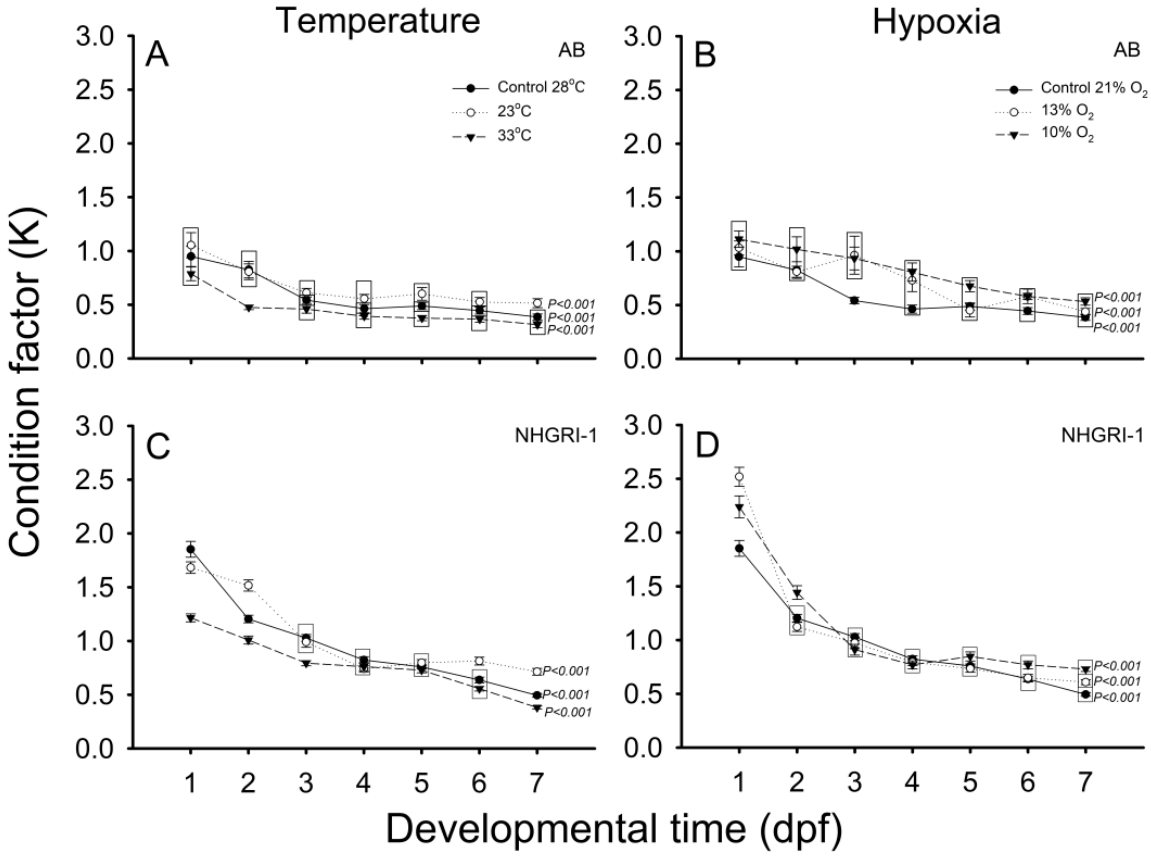

Figure 1. Condition factor (*K*) of AB (Panels **A**,**B**) and NHGRI-1 (Panels **C**,**D**) *Danio rerio* during temperature (Panels **A**,**C**) and hypoxia (Panels **B**,**D**) exposure experiments. Boxes surround mean values that are not significantly different from each other on any given developmental day. *p* values for each experimental group at the right of the figure indicate whether significant differences exist across time. $n = 15$ for each data point.

3.5. Specific Growth Rate

The specific growth rate in control larvae ranged from ~13 to ~18% day^{-1} in AB embryos and larvae and from ~4 to 27% day^{-1} in NHGRI-1 fish. In AB larvae, *SGR* from 2 to 5 dpf was lower in low-temperature embryos and larvae compared to controls ($p < 0.001$; Figure 2A). In contrast, from 1 to 2 to 4 dpf, high-temperature embryos and larvae had significantly ($p < 0.001$) higher *SGR* than controls (Figure 2A). No significant ($p > 0.05$) differences occurred at 6 and 7 dpf. Both moderate and severe hypoxia AB embryo and larval groups from 2 to 5 dpf exhibited significantly ($p < 0.001$) lower *SGR* than in controls (Figure 2B). No significant ($p > 0.05$) differences occurred at the end of the experiment on 7 dpf. NHGRI-1 embryos and larvae from both low- and high-temperature groups showed significantly ($p < 0.001$) higher *SGR* than controls from 2 to 6 dpf. By 7 dpf, low-temperature larvae showed significantly ($p < 0.001$) higher *SGR* (35.0 $\pm$ 0.6% day^{-1}) than control and high-temperature larvae (both 27 $\pm$ 1% day^{-1}; Figure 2C). The hypoxic larvae group had significantly ($p < 0.001$) increased *SGR* compared to controls throughout development (Figure 2D).

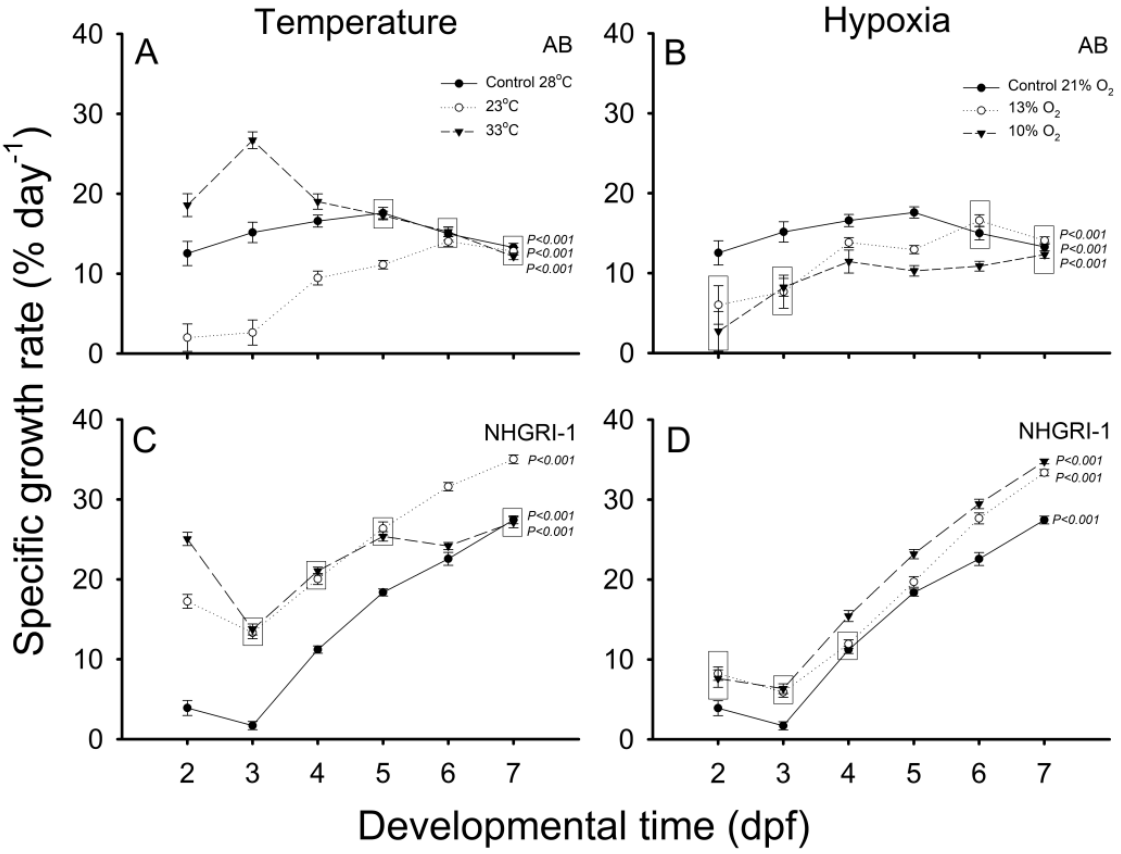

Figure 2. Specific growth rate (*SGR*) of AB (**A**,**B**) and NHGRI-1 (**C**,**D**) *Danio rerio* during temperature (**A**,**C**) and hypoxia (**B**,**D**) experiments. Boxes surround mean values that are not significantly different from each other on any given developmental day. *p* values for each experimental group at the right of the figure indicate whether significant differences exist across time. $n = 15$ for each data point.

3.6. Coefficients of Variation

The coefficient of variation for the yolk–chorion ratio ranged from 0.06 to 0.12 during early development in control embryos (Table 2). YC_R was significantly ($p < 0.001$) lower in NHGRI-1 embryos compared to AB embryos at 1 dpf in control (0.06 ± 0.01 and 0.08 ± 0.02, respectively; Z_{calc} 0.86962 > Z_{tab} 0.80785) and severely hypoxic groups (0.10 ± 0.02 and 0.14 ± 0.03, respectively; Z_{calc} 0.88340 > Z_{tab} 0.81057). NHGRI-1 embryos from moderate hypoxia had lower C_V compared to controls (0.13 ± 0.02 and 0.21 ± 0.04, respectively; Z_{calc} 1.26392 > Z_{tab} 0.89617; Table 2).

Table 2. Coefficient of variation (C_V) for morphological variables presented in Table 1 in AB and NHGRI-1 lines of zebrafish (*Danio rerio*). Asterisks on values in bold represent differences from controls at any given day. Data per day and experimental group are presented as mean ± SEM ($n = 15$). *p* values after the day 7 entry indicate differences within each experimental group across developmental time.

TEMP (°C)	O_2 (mg L^{-1})	AGE (dpf)	Yolk/Chorion Ratio		Body Mass		Embryo Mass		Total Length	
			WT AB	NHGRI-1	WT AB	NHGRI-1	WT AB	NHGRI-1	WT AB	NHGRI-1
28	7.8	1	0.08 ± 0.02	**0.06 ± 0.01** *	0.10 ± 0.02	**0.05 ± 0.01** *	0.08 ± .02	0.07 ± 0.01	0.09 ± 0.02	**0.05 ± 0.01** *
		2	0.14 ± 0.02	0.12 ± 0.02	0.14 ± 0.03	**0.07 ± 0.01** *	0.11 ± 0.02	**0.07 ± 0.01** *	0.11 ± 0.02	**0.03 ± 0.01** *
		3	0.13 ± 0.02	-	0.12 ± 0.02	**0.06 ± 0.01** *	0.14 ± 0.03	**0.06 ± 0.01** *	0.06 ± 0.01	**0.04 ± 0.01** *
		4	-	-	0.11 ± 0.02	**0.4 ± 0.02** *	0.2 ± 0.03	**0.05 ± 0.01** *	0.08 ± 0.02	**0.05 ± 0.01** *
		5	-	-	0.13 ± 0.02	**0.05 ± 0.01** *	0.14 ± 0.03	**0.05 ± 0.01** *	0.09 ± 0.02	**0.04 ± 0.01** *
		6	-	-	0.18 ± 0.08	**0.08 ± 0.02** *	0.18 ± 0.03	**0.09 ± 0.02** *	0.09 ± 0.02	**0.05 ± 0.01** *
		7	-	-	0.14 ± 0.02	**0.05 ± 0.01** *	0.14 ± 0.03	**0.06 ± 0.01** *	0.09 ± 0.02	**0.05 ± 0.01** *

Table 2. *Cont.*

TEMP (°C)	O_2 (mg L^{-1})	AGE (dpf)	Yolk/Chorion Ratio		Body Mass		Embryo Mass		Total Length	
			WT AB	NHGRI-1	WT AB	NHGRI-1	WT AB	NHGRI-1	WT AB	NHGRI-1
		1	0.11 ± 0.02	0.10 ± 0.02	0.10 ± 0.02	**0.06 ± 0.01** *	0.21 ± 0.04	**0.08 ± 0.02** *	0.13 ± 0.02	**0.04 ± 0.01** *
		2	0.15 ± 0.03	0.12 ± 0.02	0.09 ± 0.02	**0.07 ± 0.01** *	0.13 ± 0.03	**0.07 ± 0.01** *	0.10 ± 0.02	**0.04 ± .01** *
		3	0.15 ± 0.03	0.12 ± 0.02	0.11 ± 0.02	**0.08 ± 0.01** *	0.18 ± 0.03	**0.09 ± 0.02** *	0.08 ± 0.01	**0.05 ± 0.01** *
23	7.8	4	0.14 ± 0.03	-	0.07 ± 0.01	0.08 ± 0.01	0.13 ± 0.02	**0.08 ± 0.01** *	0.08 ± 0.02	**0.04 ± 0.01** *
		5	-	-	0.07 ± 0.01	0.10 ± 0.02	0.10 ± 0.02	0.10 ± 0.02	0.13 ± 0.02	**0.05 ± 0.01** *
		6	-	-	0.13 ± 0.02	**0.06 ± 0.01** *	0.15 ± 0.03	**0.06 ± 0.01** *	0.09 ± 0.02	**0.05 ± 0.01** *
		7	-		0.12 ± 0.02	**0.06 ± 0.01** *	0.13 ± 0.02	**0.06 ± 0.01** *	0.10 ± 0.02	**0.06 ± 0.01** *
		1	0.17 ± 0.03	0.20 ± 0.04	0.10 ± 0.02	**0.06 ± 0.01** *	0.14 ± 0.02	**0.07 ± 0.01** *	0.10 ± 0.02	**0.04 ± 0.01** *
		2	-	-	0.09 ± 0.02	**0.06 ± 0.01** *	0.11 ± .0.02	**0.06 ± 0.01** *	0.05 ± 0.01	0.04 ± 0.01
		3	-	-	0.10 ± 0.02	0.08 ± .0.02	0.11 ± 0.02	**0.08 ± 0.01** *	0.09 ± 0.02	**0.04 ± 0.01** *
33	7.8	4	-	-	0.13 ± 0.02	**0.05 ± 0.01** *	0.14 ± 0.03	**0.06 ± 0.01** *	0.09 ± 0.02	**0.05 ± 0.01** *
		5	-	-	0.11 ± 0.02	**0.07 ± 0.01** *	0.10 ± 0.02	**0.07 ± 0.01** *	0.12 ± 0.02	**0.04 ± 0.01** *
		6	-	-	0.15 ± 0.03	**0.05 ± 0.01** *	0.14 ± 0.03	**0.05 ± 0.01** *	0.10 ± 0.02	**0.04 ± 0.01** *
		7			0.11 ± 0.02	**0.07 ± 0.01** *	0.11 ± 0.02	**0.08 ± 0.01** *	0.12 ± 0.02	**0.04 ± 0.01** *
		1	0.15 ± 0.03	0.20 ± 0.04	0.12 ± 0.02	0.09 ± 0.02	0.26 ± 0.05	**0.10 ± 0.02** *	0.07 ± 0.01	**0.04 ± 0.01** *
		2	0.12 ± .02	0.16 ± 0.03	0.11 ± .0.02	**0.05 ± 0.01** *	0.19 ± 0.03	**0.07 ± 0.01** *	0.09 ± 0.02	**0.04 ± 0.01** *
		3	0.21 ± 0.04	**0.13 ± 0.02** *	0.10 ± 0.02	**0.07 ± 0.01** *	0.24 ± 0.04	**0.07 ± 0.01** *	0.19 ± 0.04	**0.10 ± 0.02** *
28	4.9	4	-	-	0.10 ± 0.02	**0.06 ± 0.01** *	0.09 ± .0.02	**0.06 ± 0.01** *	0.18 ± 0.03	**0.05 ± 0.01** *
		5	-	-	0.10 ± 0.02	**0.07 ± 0.01** *	0.10 ± 0.02	0.08 ± 0.01	0.11 ± 0.02	**0.05 ± 0.01** *
		6	-	-	0.09 ± 0.02	0.08 ± 0.01	0.14 ± 0.03	**0.08 ± 0.01** *	0.07 ± 0.01	0.05 ± 0.01
		7	-	-	0.12 ± 0.02	**0.05 ± 0.01** *	0.12 ± 0.02	**0.05 ± 0.01** *	0.09 ± 0.02	**0.04 ± 0.01** *
		1	0.14 ± 0.03	**0.10 ± 0.02** *	0.09 ± 0.02	0.09 ± 0.02	0.14 ± 0.02	**0.08 ± 0.02** *	0.09 ± 0.02	**0.04 ± 0.01** *
		2	0.14 ± 0.03	0.13 ± 0.02	0.09 ± 0.08	0.07 ± 0.01	0.19 ± 0.03	**0.09 ± 0.02** *	0.16 ± 0.03	**0.07 ± 0.01** *
		3	0.18 ± 0.03	0.26 ± 0.05	0.09 ± 0.02	**0.06 ± 0.01** *	0.13 ± 0.02	**0.07 ± 0.01** *	0.15 ± 0.03	**0.06 ± 0.01** *
28	3.7	4	-	-	0.12 ± 0.02	**0.07 ± 0.01** *	0.22 ± 0.04	**0.08 ± 0.01** *	0.14 ± 0.03	**0.05 ± 0.01** *
		5	-	-	0.08 ± 0.02	0.07 ± 0.01	0.12 ± 0.02	**0.07 ± 0.01** *	0.10 ± 0.02	**0.06 ± 0.01** *
		6	-	-	0.10 ± 0.02	**0.07 ± 0.01** *	0.13 ± 0.02	**0.07 ± 0.01** *	0.12 ± 0.02	**0.05 ± 0.01** *
		7	-	-	0.12 ± 0.02	**0.04 ± 0.01** *	0.13 ± 0.02	**0.03 ± 0.01** *	0.08 ± 0.01	**0.04 ± 0.01** *

NHGRI-1 larvae from most developmental days in all experimental groups had a significantly ($p < 0.001$) lower C_V compared to AB embryos for *BM*, *EM*, L_T (Table 2), *K*, and *SGR* (Figures 3 and 4). Only 36 of 187 of quantified endpoints showed any significant differences in C_V between fish lines (Table 2; Figures 3 and 4).

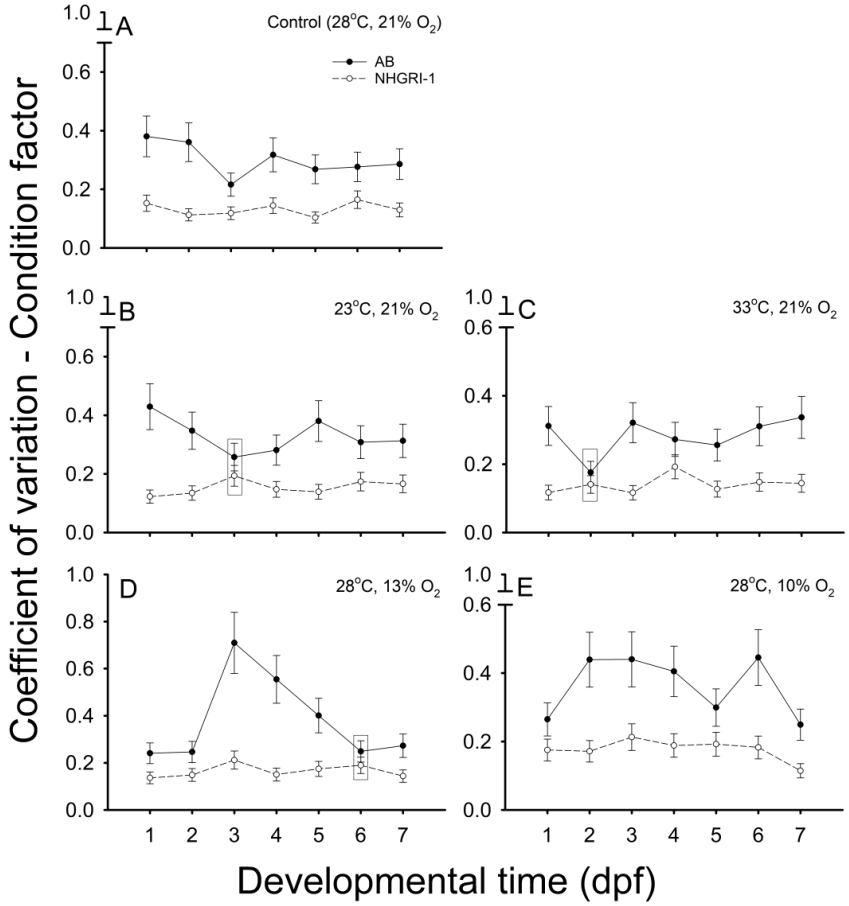

Figure 3. Coefficient of variation (C_V) for condition factor (*K*) between AB and NHGRI-1 *Danio rerio* within experimental groups throughout larval development. (**A**) Control; (**B**) low temperature; (**C**) high

temperature; (**D**) moderate hypoxia; and (**E**) severe hypoxia. Boxes surround mean values that are not significantly different from each other on any given developmental day. *p* values for each experimental group at the right of the figure indicate whether significant differences exist across time. $n = 15$ for each data point.

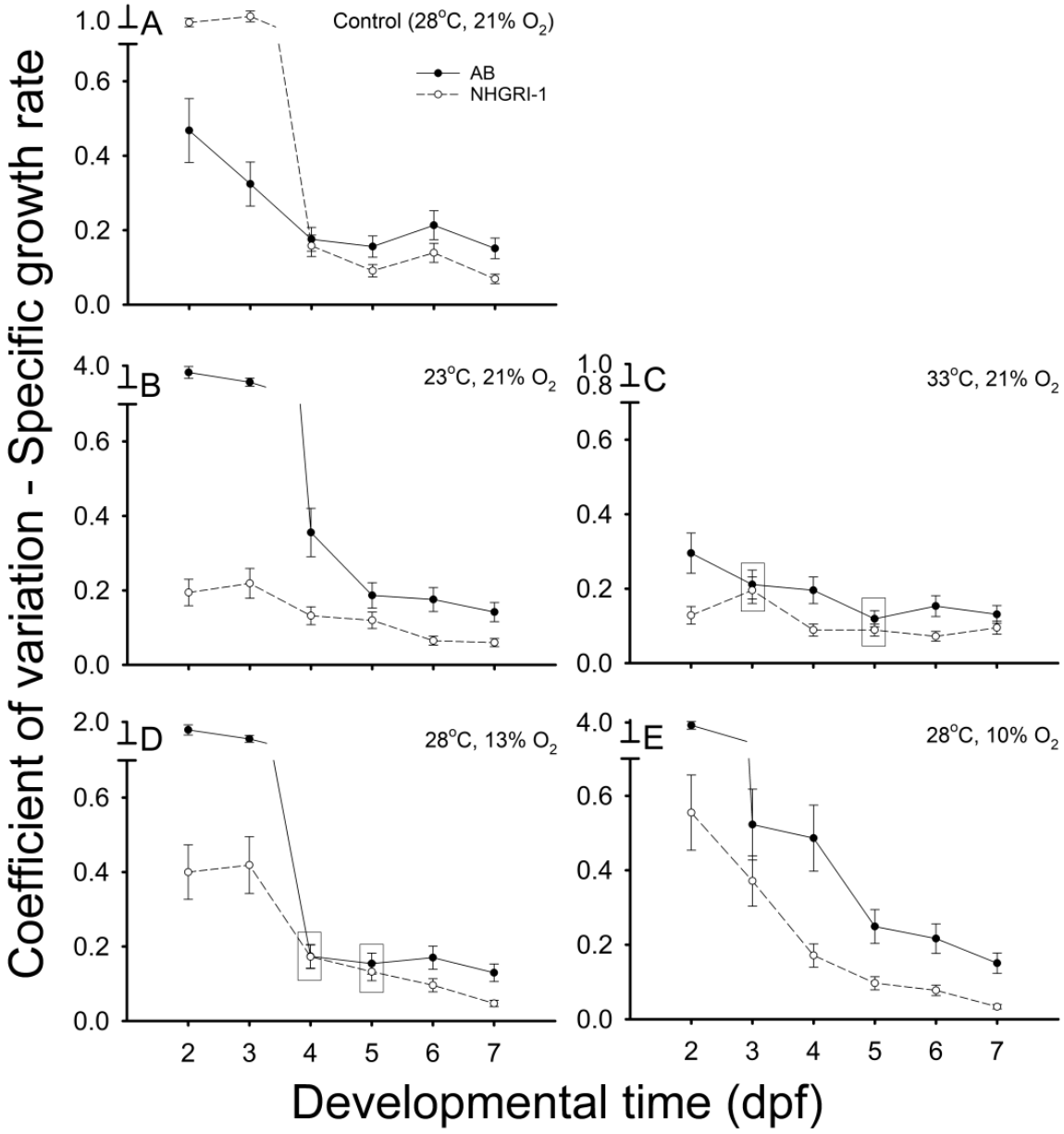

Figure 4. Coefficient of variation (C_V) for specific growth rate (*SGR*) for AB and NHGRI-1 *Danio rerio* within experimental groups throughout larval development. (**A**) Control, (**B**) low temperature; (**C**) high temperature; (**D**) moderate hypoxia; and (**E**) severe hypoxia. Boxes surround mean values that are not significantly different from each other on any given developmental day. *p* values at the right of the figure for each experimental group indicate whether significant differences exist across time. $n = 15$ for each data point.

4. Discussion

4.1. Animal Models and Variation

The genetics of animal models per se represent one of the most important factors that influence variability in experimental biology [3,8]. The genetic (and epigenetic) background of experimental subjects can modulate individual and group responses during experimentation, and its correct understanding may facilitate the interpretation and reproducibility of the results [4,6,7,9]. As an additional tool, highly inbred strains/lines have been developed to reduce genetic variability and minimize the effect of genetic backgrounds on assessed variables.

The nematode *Caenorhabditis elegans* has become a suitable model for assessing the genetic basis of quantitative traits in both natural and laboratory populations [40]. Variation among wild-type *C. elegans* isolates has been documented [41–43], and the most diverged wild-type strains (N2 and CB4856) show variation in one SNP every 840 bp in their nuclear genome [44,45]. Genetic variation between wild-type and recombinant inbred *C. elegans* lines promotes differences in maternal hatching and lifespan after dietary restriction [45,46]. Moreover, responses to different compounds (e.g., ethanol [47] and protein abundance [48]) vary among genetic variants from inbred *C. elegans* strains. Furthermore, homozygous *C. elegans* do not suffer from inbreeding depression, but outbreeding depression is commonly observed after crossing of inbred lines. This suggests that inbreeding is a naturally occurring process in *C. elegans* [49–51]. In contrast to *C. elegans*, *C. ramanei* (an outcrossed species) holds higher nucleotide variation [52,53]. These data suggest that variation is widely spread within the same taxon and genus, and even among populations of the same species.

Compared to *Caenorhabditis*, *Drosophila* possesses relatively greater variation among wild-type strains [54]. However, the employment of the two models underlies different types of investigation. Research regarding genetic variation in *C. elegans* includes the simplification of quantitative genetic studies to enhance the understanding of gene–phenotype associations across inbred individuals [55]. In contrast, studies in *Drosophila* are generally targeted to reveal complex trait variation and evolutionary dynamics [56]. The comprehensive genetic diversity in *Drosophila* influences complex traits and phenotypic variation, and contributes to its ability to adapt to different environments [56–58]. Although some studies have focused on mutations, quantitative genetic variation, and the exploration of mechanisms underlaying variability [58,59], other studies have revealed significant variation among different *Drosophila* genotypes. Differences have been observed in immune response and pathogen susceptibility, macronutrient tolerance, the modulation of apoptosis pathways, and quantitative trait loci [56,59–61].

In vertebrates, studies have shown significant differences in genetic backgrounds from wild vs. captive animals of various species [62]. Natural and captive populations of Elliot's pheasant *Syrmaticus ellioti* exhibit varying nucleotide and haplotype diversity that reflects higher variation in wild animals [63]. The African clawed frog *Xenopus laevis* and Axolotl *Ambystoma mexicanum* show contrasting evidence regarding the importance of the correct understanding of genetic backgrounds. *X. laevis* exhibited little genetic variation from wild populations compared to an isolated population in a novel environment for ~50 years [64]. In contrast, different degrees of heterozygosity and total number of single nucleotide variants (SNVs) occurred in *A. mexicanum* from captive (domesticated) and wild populations [65]. In these cases, experimental outcomes may be influenced by genetic variability, which could mask key findings during the interpretation of the results.

Mice are one of the most commonly used animal models in biomedical research. The genetic quality (and background) of mice strains is an important factor to consider for enhancing experimental outcomes [66]. Higher levels of polymorphism in wild-derived mice have been described compared to classical inbred strains [67]. Numerous examples have shown how genetic variation among mice strains (wild, inbred, and outbred) modulates their biology. Examples include studies regarding developmental progression of psoriasis disease [68]; vocal signature [69]; physical performance and anxiety-like behavior [70]; microbiome composition; [71]; lumen narrowing during vascular remodeling [72]; copy

number variation [73]; and phenotypic robustness in response to environmental variation [74], among other traits. Furthermore, different epigenetic states and transcriptional outcomes depend on genetic variation in the ribosomal DNA in multiple strains [75].

4.2. The Zebrafish Model for Studying Mechanisms for Morphological Variation

Examples of inbred fish strains and lines include the clean livebearer *Poeciliopsis lucida* [76–78], species of *Xiphophorus*—platyfish and swordtails [79,80], and the Japanese medaka *Oryzias latipes*, in which strains have been inbred for more than 100 generations [81]. Inbreeding promotes stronger long-term effects, primarily in reproduction (e.g., reduction in fecundity) or sex-ratio skewing, with growth performance being less affected [25,82,83]. However, variation in growth performance occurs in inbred zebrafish [19].

The need for a zebrafish line with a less-variable genetic background led to the production of the NHGRI-1 line [7,30]. Most studies with NHGRI-1 zebrafish address genetic/molecular research [30,84–87]. However, in the current study, we have employed this line to explore sources of morphological variation in the early developmental stages.

Despite the vast changes in fish larval morphology throughout development, embryos and early larvae show remarkable similarities among species (i.e., enlarged body, continuous fin fold, etc.) [88]. These similarities may suggest that the early life stages of fish cope with similar developmental constraints, including those for foraging, metabolism, and survival [88,89]. For example, the nutritional regime in adult zebrafish modulates epigenetically inherited patterns and gene expression in the offspring [90–93]. Furthermore, the nutritional regime determines hatching rate and larval growth performance [94–96]. A reduction (or lack) in feeding affects fishes' metabolic rate, survival, growth, and fitness [97]. Moreover, irregular feeding promotes size heterogeneity in juvenile zebrafish [98]. Additionally, most changes (morphological, physiological, behavioral, etc.) in larval fish occur within the first days of exogenous feeding [99–101]. However, the zebrafish larvae in the current study were not fed to minimize sources of variation as it occurs in other species under poor conditions, increased competition for resources, or behavioral interactions [35,102–104].

4.3. Comparison of Variation in Wild-type and Inbred Zebrafish Embryos and Larvae

Of the six variables that we evaluated, 151 of 187 endpoints showed lower variation in NHGRI-1 zebrafish, as evident from their low coefficients of variation. Additionally, morphological characters in NHGRI-1 showed less variability throughout the measurement period than in AB fish. Consequently, fewer significant differences were observed in AB fish because of the higher variation in their data, evident as overlaps of the SEMs. Variable developmental progression and growth has been observed in zebrafish [105], especially during the embryonic stage [106]. This variation in developmental progression may have contributed to increased variation among NHGRI-1 compared to AB fish, where *SGR* showed a slight increase instead of a slight decrease.

The body mass in AB fish increased ~30 µg from 1 to 7 dpf, while for NHGRI-1 fish increased only 10 µg. This result suggests that *BM* in fish early stages changes slightly because of the transfer of yolk to the embryo, as reported for other species [107,108]. Of note, *SGR* decreased after 5 dpf in AB fish in the control and higher-temperature groups. In contrast, NHGRI-1 fish from higher temperatures had a steady *SGR* from 5 to 7 dpf, but controls continued to increase. The decrease in *EM* (and *SGR* in consequence) may be related to the effect of temperature to accelerate metabolism [105,109], suggesting that fish at higher temperatures depleted yolk reserves and lacked a source of energy to sustain growth, as observed in lower-temperature and hypoxic groups. These results, together with food deprivation, may indicate that the change from feeding on egg yolk to exotrophy occurs earlier in development when temperature is increased, and food sources are needed to promote growth [108]. In addition, development was delayed by hypoxia in AB fish. Considering *EM* at 1 and 7 dpf, AB fish exposed to moderate hypoxia showed an increase of ~160% compared to controls (~150%) and fish from severe hypoxia (136%).

These changes translated into a ~12% growth (*SGR*) in the three groups by 7 dpf. However, hypoxic fish showed decreased *SGR* during the previous days. These results are consistent with the fact that hypoxia decreases embryonic growth and developmental progression in zebrafish [110–113]. Contrarily, hypoxic NHGRI-1 fish showed increases in *EM* of ~188% in fish exposed to moderate hypoxia, 175% in fish from severe hypoxia, ~127% in controls, and had higher growth rates compared to controls, especially after 5 dpf. These increases in *EM* led to increased *SGR* in hypoxic fish, compared to controls. *Breakdance* (*bre*) zebrafish mutants showed enhanced connective oxygen transport after the improvement of their cardiac performance, even though this line is known to possess decreased cardiac performance, compared to wild-type fish [114]. This characteristic ensured optimized oxygen and nutrient delivery, which increased fishes' survival. It is suggested by Kopp et al. [114] that the optimized energy metabolism in hypoxic *bre* zebrafish may be a determinant for highly energy-demanding processes including organogenesis, growth, and activity. It may be possible that the hypoxic NHGRI-1 fish in the current study could optimize their energy budget similarly to *bre* fish, especially because they show differences in cardiac function (e.g., lower heart rate) compared to the AB line [115].

Zebrafish exhibit determinate growth because of the lack of hyperplasia in juveniles and adults [116]. In fact, post-larval zebrafish treated with growth hormones showed no modification in growth rate [117]. The relationship between fish size and development is temperature- and growth rate-dependent in wild-type zebrafish [105], zebrafish mutants [118], and other fishes [119–121]. It is equally important to assess the 'relative' health of the fish through the implementation of indexes such as *K* [35]. In the current study, *K* showed very few differences between AB fish across the experiment, but it decreased from ~1 to ~0.5. Decreased *K* has been observed in fish right after hatching and then increases after first feeding [122,123]. These changes were attributed to food deprivation and nonlinear allometric length–weight relationships. Moreover, this change can occur because of developmental progression and yolk depletion, which significantly increases length, while body mass shows slight changes [108]. NHGRI-1 fish also showed decreased *K* across time (from ~2.5 to ~0.5); however, significant differences were observed between groups, especially in the temperature experiment and between fish from the control and severe hypoxia treatment. Measurements of *K* are useful to compare fish of about the same size [35]. In the current study, fish from different groups within each line showed comparable sizes. Of particular interest is that decreased variation in *K* from NHGRI-1 fish enabled the observation of differences between groups, as described above.

5. Conclusions

NHGRI-1 zebrafish embryos and larvae showed decreased variation in morphological responses to low and high temperatures and moderate and severe hypoxia. This low variation may result from the lower heterozygosity of this inbred line compared to AB fish. Despite major developmental changes occurring across time, the low variation in temperature and oxygen responses in every variable enabled the quantification of significant differences among individuals of the NHGRI-1 line. These differences were difficult to observe in AB fish because of their relatively high genetic variation, promoting the occurrence of a wider range of phenotypes. Of note, the coefficient of variation was lower in NHGRI-1 fish compared to the AB line. With these data, our results indicate that using a well-described inbred zebrafish line like NHGRI-1 can facilitate the understanding of environmentally modulated responses that could be masked by increased genetic variation. Finally, reproducibility and replicability may be improved if, in addition to providing detailed information about experimental designs and conditions, the research uses appropriate animal models matched to specific research questions.

Future research regarding low variability in morphological traits in NHGRI-1 zebrafish is warranted, especially during the development of certain characters (e.g., fin growth, pigmentation pattern, etc.) under a climate change scenario. For example, lower temperatures promote morphological changes in zebrafish at somewhat larger post-larval sizes,

while higher temperatures lead to changes in a wide range of sizes [105]. It would be interesting to assess whether the low morphological variation observed in the current study is observed at other levels of organization (e.g., physiological, behavioral, and molecular).

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